CELL TYPES AND CLOTTING REACTIONS IN THE ECHINOID, MELLITA QUINQUIESPERFORATA

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Introduction

Most of the investigations of the cellular elements and morphology of clotting in Echinoidea have been done on sea-urchins and heart-urchins (Geddes, 1880; Cuénot, 1891; Kindred, 1921, 1924) with an occasional reference to sand-dollars (Kindred, 1924), but none to *Mellita quinquiesperforata*. It is the object of this work to study the cellular elements in the perivisceral fluid of *Mellita* and to determine the sequence of events in clotting under normal and experimental conditions.

The factors which will induce or inhibit clotting in the blood of vertebrates have been the object of numerous investigations, but little work of this nature has been done on invertebrate blood. The work of Donnellon (1938) on *Arbacia* is the only one which has considered experimentally the factors responsible for agglutination of cellular elements in Echinoidea.

Material and Methods

Mellita quinquiesperforata used in this study were obtained by dredging in the coastal waters of Beaufort, N. C. The supply, renewed every other day, was kept in running sea water in laboratory tanks.

To determine the types of cells present, fluid was drained from the perivisceral cavity, a drop placed on a coverslip, inverted over a depression slide, and examined immediately under oil immersion. Camera lucida sketches were made of each cell type. Cells of each type were measured with an ocular micrometer, and their dimensions calculated from twenty of these random measurements.

In order to study phagocytosis, 1 cc. of finely granulated carmine suspension in sea water was injected by use of a hypodermic syringe through the peristomial membrane into three sections of the perivisceral cavity. After definite time intervals, one-half hour to five days, hanging-drop preparations of the perivisceral fluid were examined under oil immersion for phagocytic cells.

This same method was followed in studying fat ingestion. Hard-boiled egg yolk was ground in a mortar, and 1 cc. of a sea water suspension was injected into each animal. Olive oil was emulsified with sea water and similarly injected. Periods of three to twenty-seven hours were allowed for ingestion. One per cent osmic acid and an alcoholic solution of Sudan III were used as fat stains.

One per cent solutions of all vital stains were made up with sea water and allowed to settle. One cubic centimeter of a 1 : 100 dilution of each was injected orally into isolated animals. Hanging-drop preparations were made after periods of three to twenty-seven hours, and examined to determine which cells had stained.

To follow accurately the sequence of events in clumping, hanging-drop preparations were made and examined immediately after fluid was removed from the perivisceral cavity, and observations were continued until all cells were involved in clumps. Although clumping is a continuous process, for descriptive purposes it was possible to recognize four phases designated as stage one, two, three, and four (see p. 312). These four stages were used as criteria in judging the rapidity of the clumping of cells when salts were added to the perivisceral fluid. All salts used were Merck reagents. These were prepared in isotonic solutions. The hypertonic salt solution was prepared by adding 8 cc. of 2.5 M NaCl solution to 50 cc. of sea water.

Two cubic centimeters of perivisceral fluid were drained from an animal into a watchglass containing ½ cc. of salt solution. The clumping process was observed under high power objective of the microscope, and the number of seconds necessary for the mixture to reach each of the four clotting stages as timed by a stop-watch was recorded. At least 15 trials involving 15 animals were made with each salt solution. Controls on normal fluid were run simultaneously by substituting sea water for salt solution.

Observations

Cell Types

There are four types of amoebocytes and two varieties of leucocytes in the perivisceral fluid of *Mellita*. The amoebocytes are constantly changing shape and crawl about by formation of blunt pseudopodia. They move with a fair degree of rapidity.

Amoebocytes filled with red spherules average 20.7 μ in length and 8.6 μ in width, and are the most common type (Fig. 1). Those with colorless spherules are approximately the same size (22.9 μ long and 10 μ wide), and behave similarly (Fig. 2). Amoebocytes with variously

shaped, scattered, brown particles are present in smaller numbers than the first two types mentioned. They average 15.7 μ in length and 8.6 μ in width (Fig. 3). The brown particles in the cytoplasm exhibit continuous Brownian movement. A fourth type of amoebocyte filled with yellowish-brown spherules sometimes appears (Fig. 4). This type is more apt to be found after the sand-dollars have been kept in the laboratory for several days. Spherules similar to these are sometimes found in leucocytes with lobed pseudopodia.

There are two varieties of leucocytes; one has large hyaline ectoplasmic lobes and a small central granular endoplasmic portion (Fig. 5); the second type is spherical and is composed of many vesicular compartments, but lacks visible pseudopodia (Fig. 6). The first variety is 32.1 μ long and 27.9 μ wide; the second, 28.6 μ long and 27.9 μ wide. In the vesicles of the second variety may be seen a small amount of homogeneous pinkish material and occasionally small scattered granules which move about by Brownian movement.

Phagocytosis

One-half hour after injection of carmine into the perivisceral cavity small amounts of carmine particles are present in the amoebocytes with brown particles and in two types of leucocytes. Carmine is still present in the same cells but in much larger quantities after five days (Figs. 7, 8).

The amoebocytes with red and white spherules are similar in size, shape, and behavior, but differ in color and fat content as indicated by their reaction to osmic acid and Sudan III. It was thought that if an emulsion of egg yolk or olive oil were injected into the perivisceral cavity, amoebocytes with white spherules might be the only cells to ingest fat and they would change into amoebocytes with red or colored spherules. This was not the case; the leucocytes (Figs. 9–11) were the only cells to ingest fat.

Reaction to Vital Stains

Three hours after injection of vital stains cellular elements were examined. Safranin, sodium carminate, and methylene blue give negative results. Neutral fuchsin, however, stains the brown movable granules in the leucocytes red and similar granules in the brown amoebocytes pink. Neutral red stains the granules in both types of leucocytes red, but does not stain the granules of the brown amoebocyte. At the end of this period all cells are still viable and appear normal.

Normal Clotting

No fibrin or gelation of the plasma is observed in the normal perivisceral fluid. When the plasma is separated by filtration from the cells it remains as a clear filtrate and undergoes no visible change. Thus clotting in *Mellita* involves the cellular elements alone; all cell types, however, are involved. The first indication of clumping occurs 78 seconds after the fluid is removed from the perivisceral cavity. This phase, designated as stage 1 (Fig. 12), is characterized by clumping of a leucocyte with lobed pseudopodia either with one or two like cells or with cells of the other types. The chance meeting and adherence of other cells enlarge the clump (Fig. 13) in stage 2. In the third stage the clump becomes larger and more compact; few free cells are seen in the field. This stage is reached at an average of 145 seconds after removal from the perivisceral cavity. Occasionally an amoebocyte in which red spherules have broken down may be seen (Fig. 14*a*). Usually, however, amoebocytes with spherules or amoebocytes with brown granules continue to move about after they enter the clump. Amoebocytes with spherules are frequently seen radiating out from the clump attached only by a narrow stalk (Fig. 14*b*). It may be noticed that all the spherules are near the distal portion of this cell, and it looks as if this amoebocyte is attempting to free itself from the clump. Occasionally amoebocytes do free themselves, but they are usually retained by an adhesive substance of the leucocyte with lobed pseudopodia.

Explanation of Plate I

All figures were drawn with aid of camera lucida. × 1053.

Fig. 1. Three amoebocytes with red spherules to show differences in size and shape.

Fig. 2. Amoebocyte with colorless spherules.

Fig. 3. Amoebocyte filled with brown fluid containing scattered brown granules.

Fig. 4. Amoebocyte with yellowish-brown spherules.

Fig. 5. Leucocyte with large clear pseudopodial lobes.

Fig. 6. Spherical leucocyte without visible pseudopodial projections. Compartments may possess granules which exhibit Brownian movement as in the amoebocyte shown in Fig. 3.

Figs. 7 and 8. Leucocyte with lobed pseudopodia and spherical leucocyte showing carmine grains near the center of the cells. Taken from animals which had been injected 5 days previously.

Fig. 9. Leucocyte with pseudopodial lobes which has engulfed a droplet of olive oil stained with osmic acid.

Fig. 10. Spherical leucocyte with engulfed droplet of olive oil stained black with osmic acid.

Fig. 11. Leucocyte with lobed pseudopodia which has engulfed a bit of emulsified egg yolk stained orange with Sudan III.

Fig. 12. First stage of clotting: two leucocytes with lobed pseudopodia and an amoebocyte with red spherules.

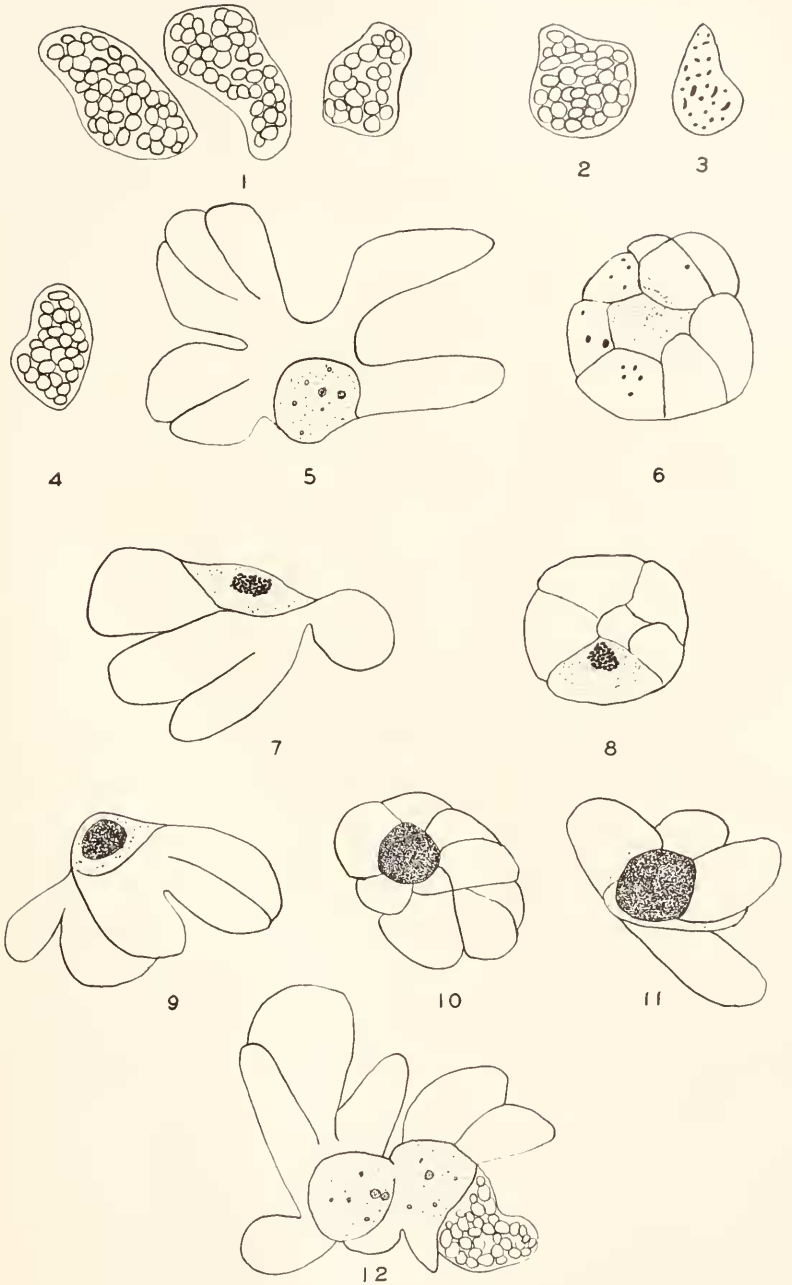

PLATE I

The clumps are large enough to be seen with the naked eye in the fourth stage. They appear as isolated, dark red, opaque masses. Microscopically it can be seen that there is a general breaking down of all cells, especially those near the center of the clump (Fig. 15). The spherules of nearly all the amoebocytes have blended into compact homogeneous masses which often appear trilobed or bilobed before becoming spherical (Figs. 15 *a, b, c*). Only occasionally can an amoebocyte with intact spherules be seen (Fig. 15*d*). The spherical leucocytes are little affected by the general contraction of the whole mass (Fig. 15*e*) but retain their identity. Since no free cells are present, this stage is considered the end-point of clotting and is reached on an average of 165 seconds after removal from the perivisceral cavity.

Action of K and Ca

In an attempt to determine the mechanism which is involved in the clotting reaction of the perivisceral fluid, certain salts were added which might influence the clotting process. The salts used were isotonic solutions of potassium chloride and calcium chloride. These were found to decrease noticeably the time required for clotting (Table I). The results show that the addition of potassium ions decreases the time of clotting much more than does the addition of calcium. When calcium is precipitated by potassium oxalate or rendered slightly ionized by sodium citrate, clotting is greatly inhibited (Table I). Potassium oxalate was more effective than sodium citrate in this respect.

Fat Solvent

A 5 per cent solution of ethyl alcohol decreases clotting time 20.6 per cent. The cells in alcohol solution reached the end-point of clotting

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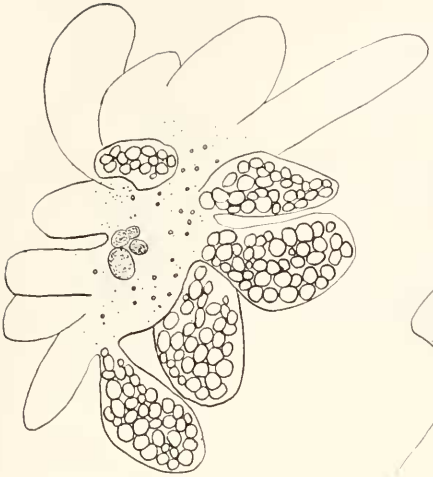
Explanation of Plate II

Fig. 13. Second stage of clotting: larger aggregation of cells including leucocytes and amoebocytes with red and white spherules.

Fig. 14. Third stage of clotting: cellular aggregation larger than in previous stage. *a*, amoebocyte in which red spherules have broken down. Cell is filled with red homogeneous material; *b*, amoebocyte with red spherules appears to be attempting to leave the cellular aggregation.

Fig. 15. Fourth stage of clotting. The cellular aggregation has contracted, most of the amoebocytes have become spherical and their spherules broken down into homogeneous masses.

R, amoebocyte with red spherules broken down; *W*, amoebocyte with white spherules broken down; *Y*, amoebocyte with yellow spherules broken down. *a, b, c*, amoebocytes whose spherules have broken down and which now appear as tri-lobed, bi-lobed, or spherical homogeneous masses; *d*, amoebocyte with colorless spherules still intact; *e*, spherical leucocyte unchanged.



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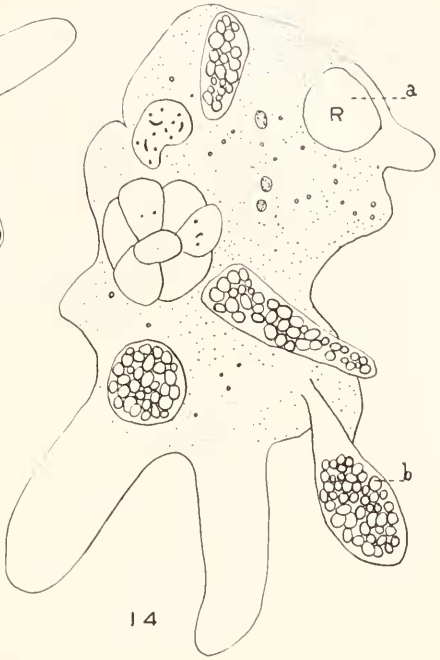

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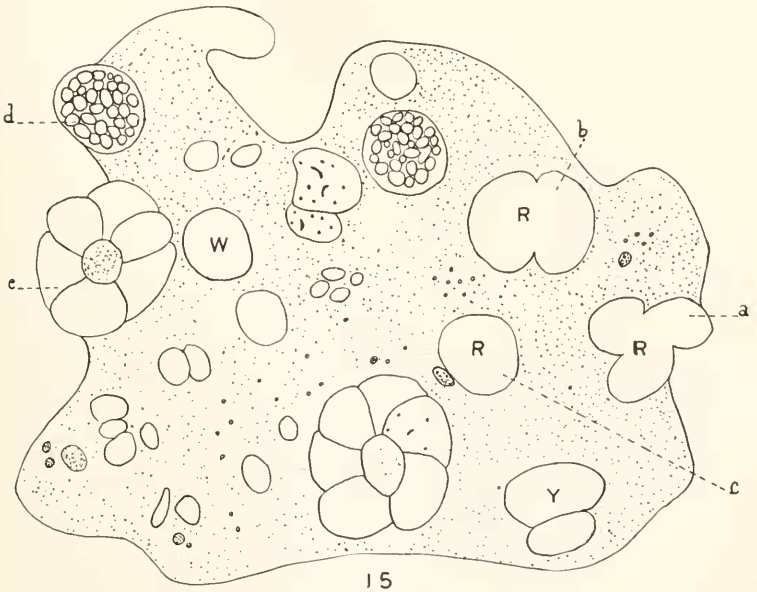

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PLATE II

135 seconds after removal from the perivisceral cavity as compared with 170 seconds in the control.

Hypertonic Salt Solution

A hypertonic salt solution accelerated clotting 45 per cent. The end-point was reached after 95 seconds as compared with 173 seconds in the control.

Action of Magnesium Sulphate

A saturated solution of $MgSO_4$ inhibited clotting indefinitely.

TABLE I

Effect of salts on clotting time of perivisceral fluid of Mellita.

Seconds required for reaction									Percentage Change in Final Clotting Time
Stage of Clotting	First Stage		Second Stage		Third Stage		Fourth Stage		
Salts	with salt	Control	with salt	Control	with salt	Control	with salt	Control	
0.53 M KCl	*		54	90	69	135	77	168	−54
0.3 M $CaCl_2$	66	72	85	89	101	130	117	165	−29
0.27 M Na Citrate	124	69	150	91	181	140	239	171	+40
0.3 M K Oxalate	129	80	151	85	169	142	275	169	+63
Hypertonic NaCl †	63	75	77	80	86	135	95	173	−45

* Too rapid to determine accurately.
† 8 cc. of 2.5 M NaCl to 50 cc. of sea water.

DISCUSSION

Various investigators (Geddes, 1880; Cuénot, 1891; Kindred, 1924) have described amoebocytes with colorless spherules, red spherules, and yellow spherules in sea-urchins. It is interesting to note that in *Mellita* there is a fourth type, a brown fluid-filled amoebocyte with scattered brown granules. This type was not found by Kindred in his comparative study of echinoderms which included the sand-dollar, *Echinarachnius eccentricus*. Geddes (1880), however, described and figured a similar type of cell in the perivisceral fluid of the heart-urchin, *Spatangus purpureus*. Brown amoebocytes as well as two types of leucocytes are phagocytic, as shown by their ability to ingest carmine grains.

They may, therefore, be important in ingestion and transportation of food particles. When the origin of the granules present in the brown amoebocytes as well as similar granules occasionally found in the vesicles of the spherical leucocyte is known, we may be able to determine whether these are food particles which are in the process of preliminary digestion or are excretory granules.

Amoebocytes with red spherules are the most common type in the perivisceral fluid of *Mellita* and Kindred (1924) reports that they are most numerous in *Strongylocentrotus franciscanus,* but less numerous in *Strongylocentrotus drobachiensis* and in *Echinarachnius eccentricus.* In the latter genus (Kindred, 1924) and in *Spatangus purpureus* and *Echinocardium* (Geddes, 1880) amoebocytes with yellow spherules are most abundant. Thus no generalization can be made as to the relative number of each type of amoebocyte in Echinoidea.

There is a theory that red and yellow amoebocytes are carriers of food. Awerinzew (1911) noticed that a variety of *Strongylocentrotus drobachiensis* which lived upon a rock and mud bottom had a greenish-yellow coloring in the skin; another variety which lived among red algae had a red appearance. Injections of ammonia-carmine and India ink revealed that amoebocytes took up these substances and deposited them in the skin. Hence Awerinzew believed that the red color in the skin and in the amoebocytes was the pigment from the red algae. The habitat of *Mellita* is sandy bottoms where there is little if any red algae, and yet the amoebocytes with red spherules are so numerous that they give the perivisceral fluid a reddish tinge. If Awerinzew's conception were correct amoebocytes would take up carmine grains, as he thought; however, this function has been found to be confined to the leucocytes by Kindred (1924) and to the leucocytes and small brown amoebocytes in our work on *Mellita.*

Tests with osmic acid substantiated by Sudan III reveal that amoebocytes with red spherules contain fat, but injection of fatty substances into the perivisceral fluid failed to reveal that it is ingested directly by any amoebocyte.

Cuénot (1891) observed vibrating cells in the perivisceral fluid of all the sea-urchins and heart-urchins he examined. These cells are not present in *Mellita* and Kindred (1924) found none in *Echinarachnius eccentricus.* In this respect the perivisceral fluid of sand-dollars which have been examined differs from that of other Echinoidea.

The presence of two varieties of leucocytes in *Mellita* is not strictly comparable to the condition found by Kindred (1924). The spherical leucocytes ("bladder-amoebocytes" of Théel, 1921) without visible pseudopodia but with vesicular compartments were not found in any

echinoderm examined by Kindred although the leucocytes with large hyaline ectoplasmic lobes present in *Mellita* were found in all classes of Echinodermata he examined. This latter variety normally possesses broad rounded lobes as Goodrich (1919) maintains, and does not normally form long filiform processes as Geddes (1880) and Cuénot (1891) described.

We have considered the spherical leucocyte a different type from the leucocyte with hyaline ectoplasmic lobes although it is possible that they may be different phases of the same cell. They are considered different types not only because of morphological differences but because the leucocyte with lobed pseudopodia is the only one to take part in the formation of a clot.

Clumping of the cellular elements in the perivisceral fluid of *Mellita* is apparently associated with the sticky exterior or adhesive secretion given off by the leucocyte with lobed pseudopodia. Amoebocytes lack this substance because when they make contact with each other they can separate, but if they come in juxtaposition with a leucocyte with lobed pseudopodia they are usually retained even though they attempt to free themselves. Schäfer (1882) described a coagulable material related to mucin which was given off by leucocytes in sea-urchins. He believed this material to be responsible for clumping. Donnellon (1938) thought that the breakdown of the red spherules of the amoebocytes in *Arbacia* liberated something which had an effect upon clotting. This may play some part, but it is very questionable whether it is important in *Mellita* because it does not occur until the end-point is nearly reached. The clot is consummated in *Mellita* on an average of 165 seconds after the perivisceral fluid has been removed from the body. The end-point of clotting in *Arbacia* (Donnellon, 1938) does not occur until 2,760 to 5,300 seconds.

Donnellon (1938) found that certain salts, such as KCl, $CaCl_2$, a hypertonic solution of NaCl, as well as fat solvents decrease the time of clotting, whereas K-oxalate and Na-citrate inhibited clotting in *Arbacia*. Using the same concentrations and molar values, we have been able to confirm Donnellon's findings. The accelerating or retarding effects were in general the same although the lengths of time involved were different. It should be noted, however, that in our work ethyl alcohol was not as effective in accelerating clotting as were the salts. The results indicate that the factors involved in clotting are somewhat similar in the two animals.

When the perivisceral fluid of *Mellita* is first removed there is little indication of clumping which denotes that leucocytes with lobed pseudo-

podia do not have a sticky exterior when in the perivisceral cavity, or if they do it is not strong enough to entrap other types of cells. Why then do these cells become sticky? It is well known that the blastomeres of developing eggs fall apart when grown in calcium-free sea water. The cement which holds the cells together is apparently due in large measure to calcium (Heilbrunn, 1937). Our experimental work indicates that when calcium ions are removed or are slightly ionized clotting is retarded. Thus calcium may be associated with the sticky substance given off by the leucocytes with lobed pseudopodia.

Our experimental work shows that factors other than calcium are important in the clotting process of *Mellita*. Howell (1912) reported that lacerated or wounded tissues emit a substance, "cephalin," which is essential to clotting in vertebrates, and Donnellon (1938) found that tissue extracts hastened clotting in *Arbacia*. In the light of these investigations it seems as though the tissue factor may be important to clotting in *Mellita*. Further experimental work may eventually determine that substances from the tissue factor release calcium from the leucocytes with lobed pseudopodia, render them sticky, and initiate clotting.

We wish to thank Dr. G. W. Wharton for collecting most of the sand-dollars used.

Summary

1. There are two types of leucocytes in the perivisceral fluid of *Mellita*. The first type is characterized by large ectoplasmic pseudopodial lobes and a small granular endoplasmic center. The second type is spherical and is composed of many vesicular compartments, but lacks visible pseudopodia. Both types are phagocytic, but the first type is the only one important in the clotting process.

2. There are three types of amoebocytes filled with spherules: those with red spherules are most abundant, those with colorless spherules are common, and those with yellow-brown spherules are rare. None of these are phagocytic. A fourth type of amoebocyte, smaller than the first three types, is filled with brown fluid and contains small scattered granules which exhibit Brownian movement. This type is phagocytic.

3. Clotting involves the cellular elements of the perivisceral fluid, but does not include the plasma. As soon as the perivisceral fluid is removed from *Mellita* the leucocytes with large pseudopodial flaps produce a sticky substance to which amoebocytes or other leucocytes adhere and finally become entangled into small clumps. As the endpoint is reached there is a general contraction of the clump, entangled amoebocytes become spherical, and their spherules break down. The

end-point of clotting is reached at an average of 165 seconds after the perivisceral fluid is removed from the body.

4. KCl, hypertonic solution of NaCl, $CaCl_2$, and a 5 per cent solution of ethyl alcohol decrease the time of clotting.

5. Na-citrate and K-oxalate increase the time of clotting, and $MgSO_4$ retards clotting indefinitely.

LITERATURE CITED

Awerinzew, S., 1911. Ueber die pigmente von Strongylocentrotus droebachiensis. *Arch. de Zool. expér. et gén.*, Ser. 5: i–viii.

Cuénot, L., 1891. Études morphologiques sur les Echinodermes. *Arch. de Biol.*, **11**: 313–680.

Cuénot, L., 1891. Études sur le sang et les glandes lymphatiques dans la série animale. Partie 2, Invertébrés. *Arch. de Zool. expér. et gén.*, Ser. 2. **9**: 593–670.

Donnellon, J. A., 1938. An experimental study of clot formation in the perivisceral fluid of Arbacia. *Physiol. Zool.*, **11**: 389–397.

Geddes, P., 1880. Observations sur le fluide périviscéral des oursins. *Arch. de Zool. expér. et gén.*, **8**: 483–496.

Goodrich, E. S., 1919. Pseudopodia of the leucocytes of invertebrates. *Quart. Jour. Micros. Sci.*, **64**: 19–27.

Heilbrunn, L. V., 1937. An Outline of General Physiology. Philadelphia.

Howell, W. H., 1912. The nature and action of the thromboplastic (zymoplastic) substance of the tissues. *Am. Jour. Physiol.*, **31**: 1–21.

Kindred, J. E., 1921. Phagocytosis and clotting in the perivisceral fluid of Arbacia. *Biol. Bull.*, **41**: 144–152.

Kindred, J. E., 1924. The cellular elements in the perivisceral fluid of echinoderms. *Biol. Bull.*, **46**: 228–251.

Schäfer, E. A., 1882. Preliminary notice of an investigation into the coagulation of the perivisceral fluid of the sea-urchin (Echinus). *Proc. Roy. Soc. London.*, **34**: 370–371.

Theel, H., 1921. On amoebocytes and other coelomic corpuscles in the perivisceral cavity of echinoderms. III. Holothurids. *Arkiv. för Zoologi*, **13**: No. 25: 1–40.